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Review

CaSm Antisense Gene Therapy: A Novel Approach for the Treatment of Pancreatic Cancer

JOSEPH R. KELLEY[1], MELISSA M. FRASER[2], JOSH M. HUBBARD[1], DENNIS K. WATSON[2] and DAVID J. COLE[1]

[1]*The Department of Surgery, Medical University of South Carolina, 171 Ashley Avenue, Room 420G CSB, Charleston, South Carolina, 29425;* [2]*The Laboratory for Cancer Genomics, Hollings Cancer Center, Medical University of South Carolina, 86 Jonathan Lucas St, Charleston, South Carolina 29425, U.S.A.*

Abstract. *Pancreatic adenocarcinoma is a major clinical problem with few effective treatment options. In the United States 29,000 cases are diagnosed annually with an associated mortality rate greater than 90%. Given this dismal prognosis, a better understanding of the molecular controls that govern pancreatic cancer is clearly needed in order to develop more effective therapies. As such, our group has been actively investigating the identification and potential application of novel gene targets for this disease. We have recently identified the cancer-associated Sm-like (CaSm) oncogene, shown that it is overexpressed in 87% of human pancreatic cancer samples, and clearly demonstrated that it functions as a classic oncogene. We have also been able to show that an adenovirus expressing antisense RNA to the CaSm gene (Ad-αCaSm) is able to reduce endogenous CaSm mRNA expression and decrease anchorage-independent growth. A single intratumor injection of Ad-αCaSm extended survival in an in vivo SCID mouse model of human pancreatic cancer. To gain insight into the mechanism of Ad-αCaSm's anti-tumor effect, cell cycle studies were performed. Ad-αCaSm treatment of pancreatic cancer cells resulted in a cytostatic block with decreased G_1-phase and increased DNA content in vitro. Importantly, the combination of Ad-αCaSm with gemcitabine (an S-phase active chemotherapy) significantly extended survival time beyond either therapy alone. These studies have defined the CaSm oncogene as a novel gene target for therapy and have begun to define its potential role in the pathogenesis of pancreatic cancer.*

Correspondence to: David J. Cole, M.D., Department of Surgery, Medical University of South Carolina, 171 Ashley Avenue, Room 420Q CSB, Charleston, South Carolina, 29425, U.S.A. Tel: 843-792-4638, Fax: 843-792-3315, e-mail: coledj@musc.edu

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Pancreatic cancer is the fourth leading cause of death from malignancy in the United States and represents a significant medical problem throughout the world. The disease often presents at an advanced stage, is resistant to all forms of therapy, and as a result has one of the highest mortality rates of any cancer(1). In light of this exceedingly poor prognosis, an effort has emerged to understand the molecular biology of this disease in order to develop more effective therapies. Examination of resected pancreatic cancer samples frequently reveals hyperplastic ductules with varying degrees of dysplasia adjacent to the tumor. The recently adopted pancreatic intraepithelial neoplasia (PanIN) pathological grading system stratifies these dysplastic lesions into low, intermediate and high grades (PanIN-1, PanIN-2, and PanIN-3, respectively). PanIN-1 lesions have low malignant potential and may never convert to overt carcinoma. PanIN-2 and PanIN-3 lesions, however, display greater cytological atypia, more architectural abnormalities, and frequently evolve to invasive cancer (http:www.path.jhu.edu/pancrease_painin). Numerous studies in the last decade have identified gene alterations involved in the various stages of this molecular progression (2-4), and these gene mutations have been extensively studied as methods for early detection and as targets for gene therapy (5-7). Promising initial results have been reported for k-ras, p53 and other gene therapies but, to date, no genetic therapy has proven clinically beneficial for the treatment of pancreatic cancer (8-10). Further studies are therefore needed to provide a better understanding of the molecular controls that govern pancreatic cancer in order to develop more effective therapies. Our group has been actively investigating a novel gene therapy approach based on the cancer-associated Sm-like (CaSm) oncogene.

The CaSm oncogene

In order to identify novel gene alteration in pancreatic cancer development, we began a project using subtractive hybridization to identify differentially expressed genes between the human pancreatic cancer cell line Capan-1 and

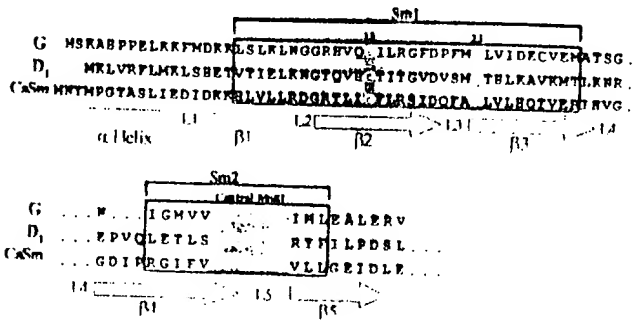

Figure 1. *Predicted structural domains of CaSm. The tertiary structure of several core Sm proteins has been resolved by X-ray crystallography. Based on these data, it is predicted that all proteins containing the Sm motifs will fold in a similar manner. The above diagram aligns the Sm motif regions of CaSm and the core Sm protein D_1 and Sm G. The positions of the predicted 5 β sheets are indicated with arrows. CaSm shares several conserved amino acids within the Sm motifs, which further indicates that CaSm will form a similar tertiary structure. Sm1 contains two invariant amino acids in all Sm containing proteins at positions 13 and 23 (highlighted in gray). Sm2 contains a central motif IRGXNI (highlighted in gray). CaSm also contains these invariant amino acids and most of the central motif in Sm2.*

the diploid, transformed but non-neoplastic pancreatic epithelial cell line HS680.PAN. The CaSm oncogene displayed a substantially higher signal in the neoplastic Capan-1 cell line compared to the normal pancreatic cells and a full-length cDNA clone was identified (11). Although the level of CaSm expression was variable between samples, fifteen of sixteen human pancreatic adenocarcinomas showed significant overexpression of a 1.2-kb CaSm mRNA in tumors relative to normal controls. This represents overexpression in 87.5% of samples (11) and is a very high frequency of oncogene overexpression in pancreatic adenocarcinoma. The frequency of CaSm involvement rivals that of k-ras mutation and is significantly more frequent than p53 mutation. Moreover, several human pancreatic cancer cell lines including AsPC-1, BXPC-3, Capan-1, Capan-2, COLO357, HPAC, MiaPaCa-2 and Panc-1 all overexpress CaSm. The CaSm mRNA was overexpressed in some samples of pancreatitis suggesting that the gene alteration may be an early event in tumor formation.

Furthermore, CaSm expression is not limited to pancreatic tumors. Cancer derived cell lines from bladder, kidney, liver, lung, ovary and rectum all overexpress CaSm mRNA (11). Although CaSm mRNA was also expressed to some degree in a variety of normal tissues, these levels of expression were markedly lower than in the corresponding tumors. Thus the oncogene is found elevated in a variety of cancer types and a low level of expression is observed in various normal tissues. This indicates that CaSm may have a normal function in a variety of cell types and that abrogation of this normal function may be a significant contributor to the pathogenesis of a variety of cancers.

Gene structure

The full-length genomic structure of CaSm has been determined in human and murine cells (Fraser and Watson unpublished data). CaSm is located on the short arm of chromosome 8 (8p11.2) between the BCL2-associated athanogene 4 (BAG4/SODD) and the steroidogenic acute regulatory protein (STAR) (www.ncbi.nlm.nih.gov Locus ID# 27257). The gene consists of 4 exons spread over a 14.5kb region (Fraser and Watson, unpublished data). The promoter region for CaSm has yet to be functionally characterized.

The CaSm gene encodes a mRNA transcript that is 1.2kbp in length with a polyadenylation signal at base pair 878-883. The translational start site is located at nucleotides 165-168. The largest open reading frame predicts a 133 amino acid polypeptide with a molecular weight of 15,179 and an isoelectric point of 4.97. CaSm was named "Sm-like oncogene" for the presence of a Sm motif in the sequence. An Sm motif is a conserved region of sequence homology that is thought to encode a region that functions in protein/protein interaction. The classical Sm motif is approximately 100bp in length and contains two Sm domains. Sm domain 1 is normally 32 amino acids long and contains a universally conserved glycine at position 13 and asparagine at position 23. There is a 10-30bp nonconserved linker region between the two Sm domains. Sm domain 2 is classically only 14 amino acids long and although highly conserved does not contain any invariant positions (12).

The CaSm sequence contains an Sm motif with two Sm domains at the expected positions (Figure 1). The CaSm Sm-1 domain is 32 amino acids long and overall, 12 of the 15 defined positions in the consensus of Sm domain 1 are conserved in CaSm including the 100% conserved glycine and asparagine residues. CaSm contains an 11 amino acid linker between its two Sm domains. Moreover, 10 out of 11 defined positions in Sm domain 2 are conserved in CaSm.

The Lsm family of proteins

Computerized BESTFIT sequence analysis of CaSm reveals a 32% identical and 60% similar homology with the human Sm G protein. This similarity led to the name "Sm-like oncogene" and may provide some information regarding the structure of CaSm(11). Sm G is part of a larger family of Sm proteins that form the spliceosome, an intricate complex of proteins that function in mRNA splicing. Recently the X-ray crystal structure of two Sm protein complexes made up of Sm D3/B

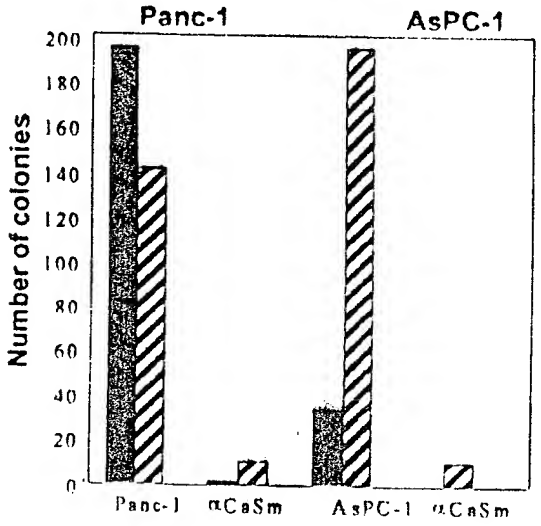

Figure 2. *CaSm antisense reduces the anchorage-independent growth of human pancreatic cancer cells. AsPC-1 and Panc-1 cells were stably transfected with CaSm antisense or a control plasmid and plated in a 1% agar solution. Cells were observed for the number and size of anchorage independent colonies 21 days after infection. The numbers of large colonies (>280μm ▨) and small colonies (140-280μm ■) are shown. Results indicate a clear decrease in both large and small colonies after treatment with CaSm antisense.*

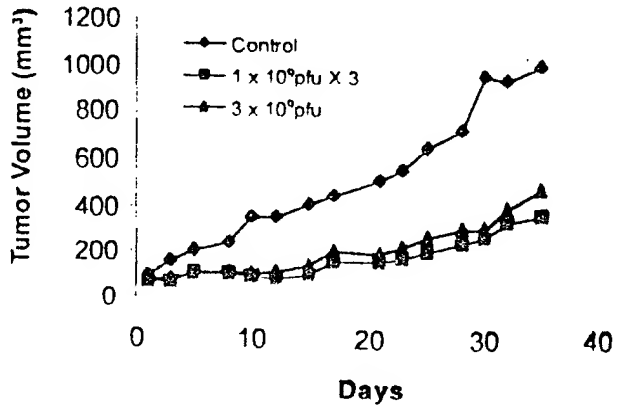

Figure 3. *Ad-αCaSm reduces subcutaneous tumor volume in SCID-Bg mice. Animals bearing subcutaneous AsPC-1 pancreatic tumors were injected on Day 0 with 100μl of saline containing Ad-LacZ (3 x 10^9pfu) or Ad-αCaSm (3 X 10^9pfu or 1 X 10^9 pfu given weekly for three weeks). Animals were monitored over time to determine the effect of this gene therapy on tumor volume. Treatment with Ad-αCaSm showed a clear decrease in tumor growth (n=10). A single large dose showed equivalent efficacy to three weekly doses.*

and Sm D1 D2 have been described(13). This structure demonstrates that Sm proteins are composed of a single helix followed by 5 anti-parallel β-pleated sheets (3 in Sm domain 1 and 2 in Sm domain 2). From this structure of the crystallized heterodimer, the β4 sheet of one protein is predicted to interact with the β5 sheet of a second protein(14). A subsequent report has expanded our knowledge of Sm protein structure and shown that the seven Sm proteins bind to one another to form a "barrel-shaped" scaffolding that is the basis of the snRNP(13). The homology between Sm G and CaSm is greatest in the Sm motifs and it seems likely that the oncogene may also form part of a "barrel-type" structure along with other Sm or Sm-like (Lsm) proteins.

While the Sm family of proteins functions in mRNA splicing, a similar family of Sm-like (Lsm) proteins plays a role in RNA decapping and degradation. Messenger RNA is normally produced in the nuclei and quickly modified by guanyl transferase, which adds a 7-methyl guanosine cap to the 5' end. Most mRNA sequences also contain an AAUAAA sequence near the 3' end. Poly-A polymerase binds to this sequence and adds a string of 50-200 adenosine residues to the end of the transcript forming a poly-A tail. The 5'-methyl guanine cap and the poly-A tail play important roles in stabilizing mRNA. Poly-A binding protein attaches to the poly-A tail of the nascent mRNA and stabilizes the message. Without a tail, the average mRNA half-life varies from less than 30 minutes to several hours. With a poly-A tail, mRNA half-lives range from several hours to even days. The 5'-methyl cap on an mRNA species also stabilizes the transcript by preventing degradation. Non-capped messages are rapidly degraded while capped mRNAs are far more stable.

The process of mRNA degradation occurs through a complex sequence of events that have been well-studied in yeast (15). The first step of the degradation process involves cleavage of the poly-A tail. In the absence of the tail, the poly-A binding protein disassociates from the mRNA transcript. Studies then indicate the Pat1 protein binds to the deadenylated mRNA and recruits a complex of Sm-like proteins(16). The Sm-like proteins (Lsm-1,2,3,4,5,6 and Lsm-7) are thought to form a seven-membered ring analogous to the "Sm-barrel" that functions in the spliceosome. The Lsm-barrel allows the decapping proteins (Dcp-1 and Dcp-2) to bind to the deadenylated mRNA transcript and cleave the 7-methyl cap (17-21). The decapped, deadenylated RNA is then rapidly degraded by the exonuclease Xrn-1(22).

Recent studies have shown that CaSm is probably a member of the Sm-like family of proteins and in fact, CaSm

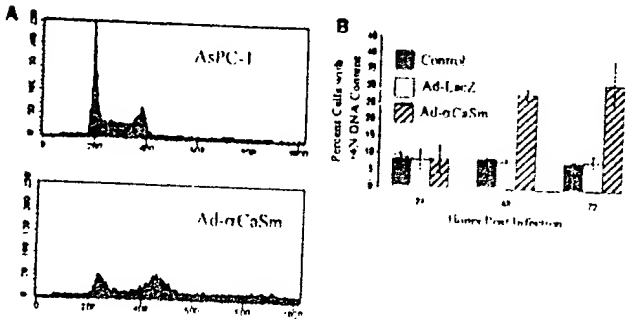

Figure 4. Ad-αCaSm increases DNA content in pancreatic cancer cells. AsPC-1 cells display a striking change in the cell cycle after infection with Ad-αCaSm (Panel A). Infected AsPC-1 contain a significant increases in >4N DNA content relative to untreated cells or Ad-LacZ-infected cells (Panel B). (Modified from Kelley JR et al. Surgery 130: 280-288, 2001).

has been designated human-like-Sm protein 1 (hLsm-1). This suggests that the oncogene may function to control mRNA stability in cancer. The yeast Lsm-1 protein (37% identical, 67% similar at the amino acid level to CaSm/hLsm-1) has also been described as Sph8 or suppressor of poly-A binding protein mutation-8 (22). As noted above, the poly-A binding protein (PABP) stabilizes mRNA and enhances transcription at the ribosome. Mutation in PABP leads to a dramatic decrease in translation that can be lethal to yeast. In this study, Sph-8 was discovered in strain of yeast that survived mutation in the poly-A binding protein. Spb8 mutants did not contain an increase in ribosome number and did not demonstrate an increased efficiency of transcription or translation. The cells were able to survive the PABP defect by increasing the stability of mRNA. Immunoprecipitation with anti-7-methyl cap antibody and Northern blot analysis indicate that Sph-8 cells accumulate deadenylated but capped mRNA transcripts. This strongly suggests that yeast Lsm-1 functions as a cofactor in the decapping process.

CaSm functions as a classical oncogene

The above studies suggest that CaSm functions in mRNA decapping and stability but further research is needed to confirm this hypothesis. The gene's role in pancreatic cancer, however, is better understood as it appears to function as a classical oncogene. CaSm transfected NIH3T3 cells form foci when plated *in vitro* (80 foci in CaSm transfectants *versus* 0 foci in untreated controls) and SCID mice injected with CaSm transfected NIH3T3 cells develop subcutaneous tumors (3/5 animals *versus* 0/10 NIH3T3 control animals. Fraser and Watson unpublished data).

More importantly, CaSm is not only expressed at a very high frequency (87% of human pancreatic adenocarcinoma samples) but is required to maintain the transformed phenotype (Figure 2). When human pancreatic cancer cell lines are transfected with a plasmid that expresses antisense RNA for CaSm, the transfectants display a striking decrease in anchorage-independent growth (11). Anchorage-independent growth in soft agar is currently the best *in vitro* correlate of *in vivo* tumorigenicity. The ability of CaSm antisense to reduce this anchorage-independent growth indicates that the gene is necessary to maintain the neoplastic state and suggests that CaSm may be useful as a gene therapy.

CaSm-based gene therapy

To test the utility of CaSm as a novel target for gene therapy, an adenoviral vector was engineered to expresses antisense RNA to CaSm (Ad-αCaSm). Northern blot analysis of human pancreatic cancer cells infected with Ad-αCaSm indicates a substantial decrease in endogenous CaSm mRNA levels after infection (23). And more importantly, Ad-αCaSm-infected cells display a significant reduction of *in vitro* proliferation compared to controls. The effect of Ad-αCaSm was examined on a panel of human pancreatic cancer cell lines including: AsPC-1, BXPC-3, Capan-1, MiaPaCa-2 and Panc-1. The proliferation of all of the cell lines is decreased following infection with Ad-αCaSm and each cell line shows a dose response (23). Reduced CaSm expression also decreases anchorage-independent growth of the panel of cell lines when plated in soft agar (23).

The effect of Ad-αCaSm on an *in vivo* model of pancreatic cancer was also examined (Figure 3). To establish this model system, AsPC-1 cells were injected subcutaneously onto the flanks of the female SCID-Bg mice. After palpable tumors developed, these animals were then treated with a single intratumor injection of saline, Ad-LacZ or Ad-αCaSm. Ad-αCaSm has a dramatic effect on tumor volume reducing tumor growth by 40%, while treatment with the Ad-LacZ control virus did not substantially alter tumor size(23).

Animals were also monitored to determine the effect of CaSm antisense on survival. Treatment with Ad-αCaSm significantly prolongs survival in this model of pancreatic cancer. Mock-infected animals all died by 35 days post treatment. Animals treated with the Ad-LacZ control virus survive for 40 days post treatment. However, treatment with Ad-αCaSm prolongs the median survival to 60 days with some animals surviving for 100 days (23). In all cases, treatment with Ad-αCaSm is well tolerated by the animals. No mice showed signs of weight loss, decreased activity, or other signs of toxicity. There was a frequent hyperemia at

the site of injection in the tumor in both control and antisense-treated animals and occasional ulceration at the injection site was noticed in both groups. Four mice from control, Ad-LacZ and Ad-αCaSm treatment groups were sacrificed 30 days after injection and examined by histology for signs of pathological change. The livers, spleens, kidneys, pancreas, and tumors were removed, fixed in formalin, embedded in paraffin and examined by hematoxylin/eosin staining. No differences were seen between control and Ad-αCaSm-treated animals in this initial study of toxicity (Kelley *et al.*, unpublished results).

The mechanism of Ad-αCaSm's anti-tumor effect

To better understand CaSm-based gene therapy a series of experiments were designed to examine underlying mechanisms involved in the anti-tumor effect. These experiments began by determining if Ad-αCaSm induced apoptosis in treated cells. Agarose gel electrophoresis, TUNEL assay and activated Caspase-3 assays all failed to detect a significant degree of apoptosis in any of the treated cell lines after infection(24). Treated cell lines were then stained with propidium iodide to determine if Ad-αCaSm induced a cytostatic effect. Results indicate a dramatic alteration in the proportion of cells in the different phases of the cell cycle. At 24 hours, CaSm antisense treatment gave a significant decrease in the number of G_1 with a corresponding increase in the proportion of S-phase cells. Forty-eight hours after infection, the G_1 population remains decreased with a corresponding increase now seen in G_2/M cells.

Interestingly, an increase in the percentage of cells with nuclei containing greater than the normal 4N content of DNA was also observed (Figure 4). At 24 hours, only 8% of control or Ad-αCaSm-infected cells display nuclei with greater than 4N DNA content. Forty-eight hours after CaSm down-regulation, this number increases to 28% (8 and 7% for untreated and Ad-LacZ controls, respectively). Seventy-two hours post infection the greater than 4N population is still present with control and Ad-LacZ-treated cells displaying 7 and 8% greater than 4N cells while Ad-αCaSm treatment yields 31%.

These results demonstrate that the predominant mechanism of Ad-αCaSm's anti-tumor effect is a cytostatic inhibition of the cell cycle. This finding of a cytostatic block with an increase in DNA content gives insight into the function of CaSm within pancreatic cancer cells and indicates an unusual anti-tumor response. There is a modest if any induction of apoptosis and the cells are not arrested in one of the classical cell cycle checkpoints. Instead, CaSm antisense-treated cells appear to re-replicate their DNA in a phenotype similar to endoreduplication. To further examine this cell cycle effect, Ad-CaSm-infected cell lines were

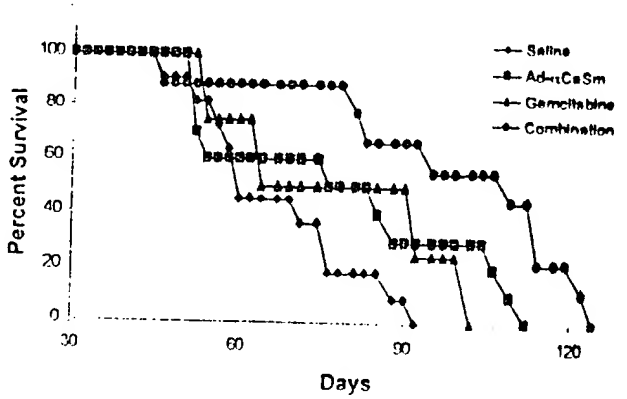

Figure 5. *Combination gemcitabine/Ad-αCaSm chemo-gene therapy significantly extends survival in a subcutaneous model of pancreatic cancer. Animals bearing subcutaneous AsPC-1 tumors were treated on Day 0 with a single intratumor injection of 100μl of saline containing Ad-LacZ or Ad-αCaSm. Animals also received 100μl intraperitoneal injections of saline or gemcitabine (40mg/kg) on Day 0, 3, 6, and 9. Animals were monitored over time to determine the effect of combination treatment on median survival. The combination chemo-gene therapy was clearly beneficial and extended median survival from 61 to 96 days. (Modified from Kelley JR et al. Surgery 130: 280-288, 2001).*

stained with DAPI and chromatin structure was examined by fluorescent microscopy. Results indicate that the chromatin of CaSm antisense-treated cells is condensed into chromosomes in the infected cells. This condensation is characteristic of prophase and shows that infected cells at least enter the first stage of mitosis. However, careful analysis of DAPI-stained cells demonstrates a striking lack of metaphase cells. In ten high-powered fields, control and Ad-LacZ-infected cells contain 17 and 19 metaphase cells, respectively. In sharp contrast, Ad-αCaSm-treated cells contain only 2 metaphase cells in 10 fields.

This data indicates that following a reduction in CaSm, cells enter the first mitotic stage of prophase but do not complete alignment in metaphase. This is an incomplete mitosis and argues that the endomitotic sequence is responsible for the increased DNA content after infection with Ad-αCaSm. In human megakaryocytes the endomitotic block occurs in an incomplete anaphase that seems to result from altered mitotic spindle dynamics. This appears to be a difference from the CaSm antisense effect. However, further experimentation is needed to more fully characterize the phase of the cell cycle where the CaSm antisense effect is predominant. We have not yet examined the spindle following infection with Ad-αCaSm. CaSm antisense may result in altered spindle dynamics that preclude metaphase alignment thus linking the two effects to a common site of defect. In addition, there are several markers of prophase,

prometaphase, metaphase and anaphase that could be used in future experiments to confirm a prophase block and study the other stages of mitosis. Experiments are currently underway to answer these questions.

Multi-modality therapy

Despite an incomplete understanding of the mechanism of Ad-αCaSm's anti-tumor effect, it is clear that decreased CaSm expression reduces pancreatic cancer cell growth in a cytostatic manner. This unique anti-tumor effect results from a decrease in G_1 phase and an increase in S-phase and DNA that immediately suggests a method of combination multi-modality therapy. An increase in the relative proportion of S-phase active cells with an accumulation of DNA suggests that a CaSm-based gene therapy may combine favorably with a cytotoxic chemotherapy that is active during synthesis. To test this hypothesis, CaSm antisense gene therapy was combined with gemcitabine chemotherapy and examined for effect on pancreatic cancer cell growth. The combination of Ad-αCaSm with gemcitabine results in a substantial decrease of *in vitro* proliferation in the AsPC-1 cell line *versus* single agent therapy (24). More importantly, this combination therapy is markedly more effective in an *in vivo* tumor model of pancreatic cancer (Figure 5). Treatment with gemcitabine in combination with an Ad-LacZ control virus reduces tumor volume by 35%. Ad-αCaSm alone decreases tumor size by 36% but the combination therapy reduces tumor volume by more than 70%(24).

Moreover, the multi-modality therapy significantly prolongs survival compared to either single agent. Untreated control animals die within 80 days in this model system with a median survival time of 60 days. Treatment with gemcitabine prolongs survival to 78 days, whereas Ad-αCaSm as a single agent produces a median survival time of 79 days. However, that combination of Ad-αCaSm with gemcitabine results in a median survival time of 100 days with some animals surviving for more than 120 days(24).

Conclusion

Pancreatic adenocarcinoma remains a major medical problem with an extremely high mortality rate. Lack of an effective therapy has led to an increased interest in novel treatment modalities to improve the management of this dismal disease. Recent studies by our group suggest that the cancer-associated Sm-like (CaSm) oncogene serves as a novel target in the pathogenesis of pancreatic cancer and that CaSm-based gene therapy may have potential. CaSm is overexpressed in more than 80% of human pancreatic cancer samples and a decrease in CaSm expression results in a decrease in pancreatic cell growth both *in vitro* and *in vivo*. The mechanism of this anti-tumor effect appears to be a cytostatic inhibition of the cell cycle with a corresponding increase in DNA synthesis activity. This mechanism of action allows Ad-αCaSm gene therapy to combine favorably with gemcitabine chemotherapy resulting in an additive decrease in tumor growth and a significant survival advantage.

Thus CaSm represents a novel target with exciting potential as a new treatment approach for pancreatic cancer. Further work is necessary to more fully describe the normal function of CaSm and the mechanism of Ad-αCaSm's anti-tumor effect. Additional studies combining CaSm-based gene therapy with other chemotherapeutic agents are currently underway as are experiments to test the utility of CaSm as an early detection.

Acknowledgements

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